

In Vitro Response to Chloramphenicol, Tetracycline, Ampicillin, Gentamicin, and Beta-Lactamase Production by Halophilic Vibrios from Human and Environmental Sources

SAM W. JOSEPH,* ROBERT M. DeBELL, AND WENDELL P. BROWN

Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland 20014

Received for publication 15 September 1977

Isolates of *Vibrio parahaemolyticus* and *V. alginolyticus* from human and environmental sources were examined for antibiotic susceptibility by the methods of minimal inhibitory concentration (MIC) in broth and agar diffusion. These strains were found to be almost uniformly susceptible to chloramphenicol and tetracycline within attainable serum levels. The relationships of zone sizes to MICs for these two antimicrobial agents and ampicillin conformed essentially to those obtained by standard methods with gram-negative rods. Most strains were resistant to ampicillin and exhibited β -lactamase activity, which accounted for this resistance. Nine of 30 *V. alginolyticus* strains from environmental sources were ampicillin resistant but did not produce measurable amounts of β -lactamase. Three strains exhibited multiresistance to high concentrations of chloramphenicol, tetracycline, and ampicillin, which suggests the presence of plasmids. Although the great majority of vibrios appeared to be susceptible to gentamicin by agar diffusion, susceptibility could not be measured by MIC because the added NaCl, required for growth by the halophilic vibrios, diminished gentamicin activity.

Vibrio parahaemolyticus, presumably of marine origin, is a cause of food-borne gastroenteritis. Its isolation in many countries suggests a global distribution (1, 6). More recently, *Vibrio alginolyticus* and lactose-fermenting vibrios, also, have been described with increasing frequency as causes of both intestinal and extraintestinal illnesses (8, 13, 15, 16; and S. W. Joseph, unpublished data). Despite their medical importance, strains of *V. parahaemolyticus* and in particular *V. alginolyticus* were not extensively tested for antimicrobial susceptibility.

An earlier report (9) included a semiquantitative study of the multiple-drug-resistant nature of a small number of clinical isolates of *V. parahaemolyticus*. In this paper we present the results of determinations performed on well-characterized *V. parahaemolyticus* and *V. alginolyticus* from both human and environmental sources. Minimal inhibitory concentration (MIC) and agar disk diffusion inhibition by four antibiotics are compared. We also report the presence of β -lactamase activity, which accounted in most cases for almost uniform resistance to ampicillin.

MATERIALS AND METHODS

Bacterial strains. The organisms were isolated originally on thiosulfate-citrate-bile salt-sucrose agar as the predominant strain derived from clinical speci-

mens or from coastal waters and seafood taken from the Java Sea. The majority of human isolates were obtained from patients with gastroenteritis symptomatology at various hospitals or polyclinics in Makassar (Udjung, Pandang) and Jakarta, Indonesia. There were also eight strains from W. Irian, and one from Palu, Sulawesi (Celebes). All strains were identified and characterized at the time of isolation, as described in the Bacteriological Analytical Manual of the Food and Drug Administration (5), and serotyped by using commercially available typing sera (3) (Koshida Kagabu Kogyo Co., Ltd., Tokyo, Japan). After reactivation from lyophilized stocks, they were recharacterized for this study.

Three strains of *V. parahaemolyticus*, ATCC 27519, 17802, and 17803, one of *Staphylococcus aureus* ATCC 25923, and one of *Escherichia coli* ATCC 25922 were included for comparison. Except where otherwise indicated, *V. parahaemolyticus* and *V. alginolyticus* from human sources are termed VPH and VAH, and those from environmental sources VPE and VAE, respectively.

Antibiotics. Chloramphenicol, tetracycline, gentamicin, and ampicillin were obtained commercially as sterile powders for susceptibility testing (Ames Co., Miles Laboratories, Inc., Elkhart, Ind.). Stock solutions were made according to the instructions of the producer and used immediately or stored frozen for less than 5 days. The susceptibility disks were commercially available chloramphenicol and tetracycline (30 μ g) and gentamicin and ampicillin (10 μ g) (Baltimore Biological Laboratories, Cockeysville, Md.).

Susceptibility testing. The MIC determinations,

performed in duplicate, were done by slight modification of standard methods (17). Brain heart infusion broth (BHI) (Difco Laboratories, Detroit, Mich.) was modified to contain 2% NaCl for testing the vibrios and was unchanged for all other organisms. In brief, BHI broth in 10-ml tubes containing various concentrations of drugs was inoculated with sufficient actively growing culture to yield a concentration of approximately 10^5 colony-forming units per ml on plate count. The cultures were incubated at 37°C, and the results were read after 20 to 24 h. The MIC was regarded as the least amount of antibiotic allowing no visible growth or at the most \leq five colonies.

Disk susceptibility testing was performed in duplicate by standard disk diffusion methods (2, 11) except that Mueller-Hinton agar (BL) was modified to contain 2% NaCl, for growing the halophilic vibrios, and the pH was carefully adjusted to 7.2 to 7.4.

β -Lactamase production. Determinations were performed according to the iodometric assay of Perret (12) as modified by Workman and Farrar (18). The organisms, from a growing culture in BHI (2% NaCl), were streaked on nutrient agar containing 0.2% soluble starch and incubated at 37°C for 20 to 24 h. The plates were flooded with 3 ml of freshly prepared phosphate-buffered saline (pH 6.4) containing iodine (3 mg/ml), potassium iodide (15 mg/ml), and aqueous penicillin G (50 mg/ml) and observed for 30 min for zones of decolorization indicating β -lactamase production.

RESULTS

The results are presented in Fig. 1 as scattergrams illustrating the relationship between the diameters of the zones of inhibition and the MICs. Also shown, in relation to these data, are the maximum serum levels attainable when these antimicrobial agents are given orally (14). In most instances, susceptibility patterns were limited to a narrow range of antibiotic concentrations, which prevented plotting meaningful regression lines. Tables 1 and 2 present percent distributions of strains of environmental and human sources of *V. parahaemolyticus* and *V. alginolyticus*, respectively, among the various susceptibility levels, as measured by MIC.

Chloramphenicol was highly inhibitory for the great majority of strains and in particular for VPE and VAE. The MICs for VPH and VAH were <12.5 μ g/ml (the maximum attainable serum level) for 95 and 79% of the strains, respectively, but a majority were inhibited by 3.1 μ g/ml. In the case of VPE and VAE, the MICs for virtually all strains were ≤ 1.6 μ g/ml. Zones of inhibition generally ranged from 19 to 33 mm in diameter. They were <18 mm in diameter in the case of only one VPH and six VAH.

Tetracycline, likewise, was highly inhibitory for the great majority of strains and especially VAE. The MIC values were similar to those obtained with chloramphenicol. Only three VPH and four VAH had MICs ≥ 12.5 μ g/ml. Zones of inhibition generally ranged from 19 to 27 mm

in diameter, and only with nine strains were the zones ≤ 18 mm in diameter.

Gentamicin was highly inhibitory when tested by agar diffusion, but, surprisingly, not by MIC. Of the VPH and VAH tested, 97 and 90%, respectively, had zones of inhibition ≤ 13 mm with a range of 13 to 25 mm. Comparable results were obtained with VPE and VAE. However, with the exception of three VPH and three VPE, which were inhibited by 6.3 μ g/ml, all strains required a concentration ≥ 12.5 μ g/ml.

Ampicillin was ineffective with most strains. With the exception of one VPH and six VAH, all strains required concentrations >12.5 μ g/ml for inhibition, and the great majority had MICs ≥ 500 μ g/ml. Results obtained by agar diffusion were in general agreement with those obtained by MIC, but a few strains, judged resistant by MIC, had inhibition zones of 15 to 18 mm in diameter. Two VAH and one VPH strains that were resistant to ampicillin were also resistant to both chloramphenicol and tetracycline.

β -Lactamase activity. Of the 160 VPH, 156 were β -lactamase producers. Two of the negative strains were susceptible to low concentrations of ampicillin (3.1 μ g/ml), and two were resistant to 500 μ g/ml. There were three β -lactamase producers that were susceptible to relatively low concentrations (one at 12.5 and two at 3.1 μ g/ml). Seventy-two of the VPE tested were positive. The five negative strains were resistant to concentrations ≥ 500 μ g/ml.

Twenty-four of the 26 VAH were β -lactamase positive. Four of these strains had MICs of 12.5 μ g/ml. The two strains negative for β -lactamase had MICs of 12.5 and <1.6 μ g/ml. β -Lactamase was observed in 21 of 30 ampicillin-resistant strains of VAE. The nine β -lactamase negative strains were resistant to ampicillin concentrations ≥ 500 μ g/ml. These results are summarized in Table 3.

DISCUSSION

These studies have been directed toward the applicability of standard antibiotic susceptibility tests to the halophilic vibrios. Chloramphenicol and tetracycline were selected for their broad-spectrum effectiveness and ampicillin and gentamicin for their usefulness in treating gastroenteritis and therapeutically difficult gram-negative rod infections, respectively. The results with tetracycline, chloramphenicol, and ampicillin show that the standard methodology recommended for these antibiotics can be used for in vitro susceptibility testing of *V. parahaemolyticus* and *V. alginolyticus*.

The results obtained with gentamicin were inconclusive because agar diffusion zone sizes

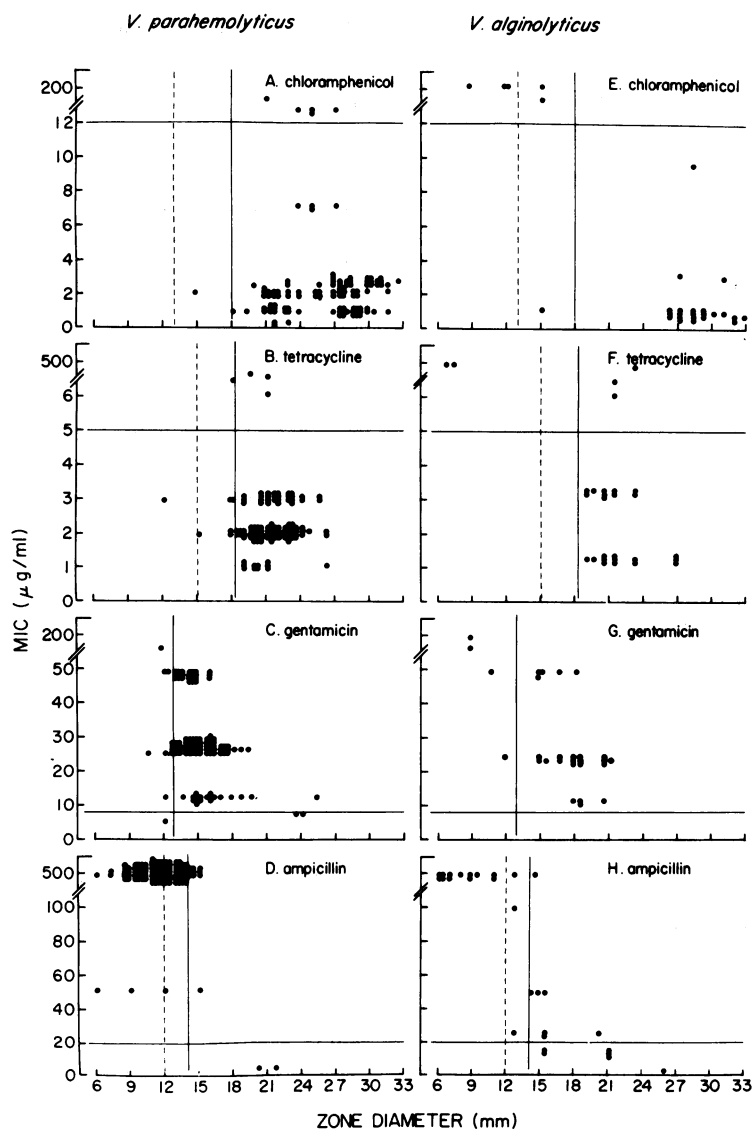


FIG. 1. Each scattergram shows the relationship between MICs and zones of inhibition for all human strains of *V. parahaemolyticus* and *V. alginolyticus* with the indicated antibiotic. The horizontal lines within each scattergram represent maximum attainable serum levels of the antibiotics. The vertical solid lines represent zone diameters that are accepted breakpoints between resistance and susceptibility of gram-negative organisms. The region between the solid and broken vertical lines encompasses those strains of intermediate susceptibility. Each point represents a single strain tested.

that indicated susceptibility did not agree with the MICs, which indicated that doses required were well above normally attained serum levels. Other studies have demonstrated that gentamicin susceptibility was diminished by increased cation concentrations (7, 10); therefore, we investigated the possibility of similar responses with these vibrios. The results, to be included in a separate report, showed that increasing concentrations of Na^+ produced a decrease in gentamicin activity against halophilic vibrios.

This study demonstrates a high incidence of ampicillin-resistant halophilic vibrios, although the isolates were from an area with distinct geographical limits. Several standard strains from Japan used in this study revealed a similar resistance pattern. There was good correlation between ampicillin resistance and β -lactamase activity in these organisms.

The frequency of β -lactamase activity observed in vibrios from nonhuman sources suggests that this resistance can be acquired in the

TABLE 1. Susceptibility of *V. parahaemolyticus* from human and environmental sources to antibiotics as determined by MIC broth technique

| Antibiotic | Source of strains | % of strains sensitive at concentration ($\mu\text{g/ml}$): | | | | | | | | | | | Median ($\mu\text{g/ml}$) |
|-----------------|-------------------|---|-----|------|------|------|-----|------|------|------|-------|--------------|-----------------------------|
| | | 0.2 | 0.4 | 0.8 | 1.6 | 3.1 | 6.3 | 12.5 | 25.0 | 50.0 | 100.0 | ≥ 500.0 | |
| Chloramphenicol | H ^a | 0.0 | 2.5 | 31.0 | 28.4 | 30.2 | 3.5 | 3.5 | 0.0 | 0.9 | 0.0 | 0.0 | 1.26 |
| | E ^b | 1.3 | 3.9 | 46.7 | 44.2 | 3.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.78 |
| Tetracycline | H | | | 9.4 | 62.0 | 25.0 | 0.9 | 0.0 | 0.9 | 0.9 | 0.9 | 0.0 | 1.43 |
| | E | 2.6 | 3.9 | 29.8 | 42.9 | 16.9 | 0.0 | 3.9 | 0.0 | 0.0 | 0.0 | 0.0 | 1.06 |
| Gentamicin | H | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.6 | 17.2 | 56.9 | 22.4 | 0.0 | 0.9 | 19.13 |
| | E | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.9 | 15.6 | 75.3 | 5.2 | 0.0 | 0.0 | 17.56 |
| Ampicillin | H | | | | | 1.7 | 0.0 | 0.0 | 0.0 | 3.5 | 0.9 | 93.9 | — ^c |
| | E | | | | | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 98.7 | — |

^a H, Human; total number, 116.^b E, Environmental; total number, 77.^c —, Precise value could not be determined.TABLE 2. Susceptibility of *V. alginolyticus* from human and environmental sources to antibiotics as determined by MIC broth technique

| Antibiotic | Source of strains | % of strains sensitive at concentration (µg/ml): | | | | | | | | | | | Median (µg/ml) | |
|-----------------|-------------------|--|------|------|------|------|-----|------|------|------|-------|-------|----------------|----------------|
| | | 0.2 | 0.4 | 0.8 | 1.6 | 3.1 | 6.3 | 12.5 | 25.0 | 50.0 | 100.0 | 200.0 | | ≥500.0 |
| Chloramphenicol | H ^a | | | | 14.3 | 57.2 | 7.0 | 3.6 | 0.0 | 0.0 | 3.6 | 14.3 | 0.0 | 2.53 |
| | E ^b | 3.3 | 23.3 | 20.0 | 53.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.85 |
| Tetracycline | H | | | | 50.3 | 31.8 | 3.6 | 3.6 | 0.0 | 0.0 | 0.0 | 0.0 | 10.7 | — ^c |
| | E | 6.7 | 20.0 | 50.0 | 20.0 | 3.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.58 |
| Gentamicin | H | | | | 0.0 | 0.0 | 0.0 | 14.3 | 57.1 | 21.4 | 3.6 | 3.6 | 0.0 | 20.30 |
| | E | | | | 0.0 | 0.0 | 3.3 | 36.7 | 50.0 | 6.7 | 3.3 | 0.0 | 0.0 | 14.50 |
| Ampicillin | H | | | | 3.6 | 0.0 | 0.0 | 17.9 | 14.3 | 10.6 | 3.6 | 0.0 | 50.0 | 300.00 |
| | E | | | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | — |

^a H, Human; total number, 28.^b E, Environmental; total number, 30.^c —, Precise value could not be determined.TABLE 3. Results of examination for β -lactamase production by *V. parahaemolyticus* and *V. alginolyticus*^a

| Organism | Source | β -Lactamase positive/total examined | % Positive |
|----------------------------|---------------|--|------------|
| <i>V. parahaemolyticus</i> | Human | 156/160 | 97.5 |
| <i>V. parahaemolyticus</i> | Environmental | 72/78 | 92.3 |
| <i>V. alginolyticus</i> | Human | 24/26 | 92.3 |
| <i>V. alginolyticus</i> | Environmental | 21/30 | 70.0 |

^a β -Lactamase production and ampicillin resistance were observed with *V. parahaemolyticus* (ATCC 27519, 17802, 17803); *V. cholerae* ATCC 25871; and *S. aureus* ATCC 13301. *S. aureus* (ATCC 25923 and 6538P) did not have detectable β -lactamase and were susceptible respectively to 0.19 and 1.56 μg of ampicillin per ml. β -Lactamase was demonstrated with *E. coli* ATCC 25922, which was susceptible to 6.25 μg of ampicillin per ml.

environment. One possible source of this acquisition was human discharge, known to have contaminated some of the collection sites. In light of the fact that R-factors exist in organisms from areas unexposed to commercial antibiotics, as shown by Davis and Anandan (4), it is possible that plasmids are transferred from human fecal organisms to naturally occurring vibrios. Also, the absence of β -lactamase activity in 30% of the VAE and some of the VPE and VPH strains

resistant to high ampicillin concentrations suggests that more than one mechanism exists for ampicillin resistance in these organisms. We are attempting to determine whether or not the β -lactamase activity and observed multiple-resistance patterns in three strains are attributable to R-factors.

In conclusion, our findings show that the criteria for standard antibiotic susceptibility tests are applicable to halophilic vibrios with three of four antimicrobial agents. These results must be confirmed by clinical trials. Observations with ampicillin resistance, β -lactamase activity, and the occurrence, in a few cases, of multiple-drug resistance emphasizes the importance of surveillance of drug susceptibilities of halophilic pathogens.

ACKNOWLEDGMENTS

We thank Deborah Wolfe and Carol Szipszky for their technical assistance. We are grateful to Emilio Weiss for reviewing the manuscript.

This work was supported by Naval Medical Research and Development Command, Research Work Unit Number ZF51524009.0057.

LITERATURE CITED

1. Barker, W. H., R. E. Weaver, G. K. Morris, and W. T. Martin. 1975. Epidemiology of *Vibrio parahaemolyticus* infections in humans, p. 257-262. In D. Schlesinger (ed.), *Microbiology*—1974. American Society for Microbiology, Washington, D.C.

2. Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 36:493-496.
3. Committee on the Serological Typing of *Vibrio parahaemolyticus*. 1970. New serotypes of *Vibrio parahaemolyticus*. *Jpn. J. Microbiol.* 14:249-250.
4. Davis, C. E., and J. Anandan. 1970. The evolution of R factor. A study of a "preantibiotic" community in Borneo. *N. Engl. J. Med.* 282:117-122.
5. Food and Drug Administration. 1972. Bacteriological analytical manual. Division of Microbiology, Food and Drug Administration, Washington, D.C.
6. Fujino, T. 1974. Discovery of *Vibrio parahaemolyticus*, p. 1-4. In T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
7. Gilbert, D. N., E. Kutscher, P. Ireland, J. A. Barnett, and J. P. Sanford. 1971. Effect of the concentrations of magnesium and calcium on the *in vitro* susceptibility of *Pseudomonas aeruginosa* to gentamicin. *J. Infect. Dis.* 124(S):37-45.
8. Hollis, D. G., R. E. Weaver, C. N. Baker, and C. Thornsberry. 1976. Halophilic *Vibrio* species isolated from blood cultures. *J. Clin. Microbiol.* 3:425-431.
9. Joseph, S. W. 1974. Observations on *Vibrio parahaemolyticus* in Indonesia, p. 35-45. In T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
10. Medeiros, A. A., T. F. O'Brien, W. E. C. Wacker, and N. F. Yulug. 1971. Effect of salt concentration on the apparent *in vitro* susceptibility of *Pseudomonas* and other gram negative bacilli to gentamicin. *J. Infect. Dis.* 124(S):59-64.
11. National Committee for Clinical Laboratory Standards. 1975. Performance standards for anti-microbial disc susceptibility tests. The National Committee for Clinical Laboratory Standards, Villanova, Pa.
12. Perret, C. J. 1954. Iodometric assay of penicillinase. *Nature (London)* 174:1012-1013.
13. Rubin, S. J., and R. C. Tilton. 1975. Isolation of *Vibrio alginolyticus* from wound infections. *J. Clin. Microbiol.* 2:556-558.
14. Sherris, J. C. 1974. Future needs, p. 439-442. In E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed, American Society for Microbiology, Washington, D.C.
15. Thorsteinsson, S. B., J. N. Minuth, and D. M. Musher. 1974. Clinical manifestations for halophilic noncholera *Vibrio* infections. *Lancet* ii:1283-1284.
16. Von Gravenitz, A., and G. O. Carrington. 1973. Halophilic *Vibrios* from extra-intestinal lesions in man. *Infection* 1:54-58.
17. Washington, J. A., and A. L. Barry. 1974. Dilution test procedures, p. 410-417. In E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
18. Workman, R. G., and W. E. Farrar. 1970. Activity of penicillinase in *Staphylococcus aureus* as studied by the iodometric method. *J. Infect. Dis.* 121:433-437.